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Dual Effects of Plant Steroidal Alkaloids on *Saccharomyces cerevisiae*†

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Many plant species accumulate sterols and triterpenes as antimicrobial glycosides. These secondary metabolites (saponins) provide built-in chemical protection against pest and pathogen attack and can also influence induced defense responses. In addition, they have a variety of important pharmacological properties, including anticancer activity. The biological mechanisms underpinning the varied and diverse effects of saponins on microbes, plants, and animals are only poorly understood despite the ecological and pharmaceutical importance of this major class of plant secondary metabolites. Here we have exploited budding yeast (*Saccharomyces cerevisiae*) to investigate the effects of saponins on eukaryotic cells. The tomato steroidal glycoalkaloid α-tomatine has antifungal activity towards yeast, and this activity is associated with membrane permeabilization. Removal of a single sugar from the tetrasaccharide chain of α-tomatine results in a substantial reduction in antimicrobial activity. Surprisingly, the complete loss of sugars leads to enhanced antifungal activity. Experiments with α-tomatine and its aglycone tomatidine indicate that the mode of action of tomatidine towards yeast is distinct from that of α-tomatine and does not involve membrane permeabilization. Investigation of the effects of tomatidine on yeast by gene expression and sterol analysis indicate that tomatidine inhibits ergosterol biosynthesis. Tomatidine-treated cells accumulate zymosterol rather than ergosterol, which is consistent with inhibition of the sterol C_{24} methyltransferase Erg6p. However, *erg6* and *erg3* mutants (but not *erg2* mutants) have enhanced resistance to tomatidine, suggesting a complex interaction of *erg* mutations, sterol content, and tomatidine resistance.

Plants produce a vast array of structurally diverse secondary metabolites. These natural products serve as attractants for agents that mediate pollination and seed dispersal; they also provide chemical defenses against pests, pathogens, and invasion by neighboring plants (47). Small molecules therefore play key roles in ecological interactions between plants and other organisms. We exploit the rich reservoir of metabolic diversity provided to us by diverse plant species in order to find new drugs and other valuable compounds. The chemical "space," in terms of the number and variety of molecules produced by plants, is enormous. Structures of over 100,000 diverse compounds have been reported so far (11), and this is inevitably just the tip of the iceberg. However, with a few well-characterized exceptions, we know very little about the biological properties of plant secondary metabolites. Characterization of the biological activities of these compounds will be critical, both from an ecological perspective and a pharmaceutical perspective.

The terpenes are one of the largest and most diverse groups of plant secondary metabolites (9). They include sterols and triterpenes, complex compounds that are formed by the cyclization of 2,3-oxidosqualene. Sterols and triterpenes can accumulate as glycoside conjugates in substantial quantities in plants. These glycosides, which include steroidal glycoalkaloids, are commonly referred to as saponins (24). Saponins have a broad range of properties that includes antimicrobial, anti-insect, and allelopathic activity, and there is good evidence that they contribute to plant defense (8, 20, 24, 30, 36, 38). They also have a range of important pharmacological applications (13, 23, 36). Examples of members of this family of plant secondary metabolites that are exploited for drug or medical use include digitonin (used for cardiovascular treatment), diosgenin (a precursor for chemical synthesis of steroid hormones), the *Quillaja* saponins (adjuvants), and avicins (new and effective anticancer agents) (13, 19, 21, 22, 24, 36). Some saponins have negative effects and are detrimental to human health. Steroidal glycoalkaloids, for example, can be toxic when ingested (16, 24).

Although it is clear that saponins have a diverse range of biological activities, very little is known about the mode of action of these compounds. Saponins form complexes with sterols and cause sterol-dependent membrane permeabilization (30). The antifungal activity of saponins is generally attributed to these membrane-permeabilizing properties. The precise mechanism of membrane disruption is unknown, but the sugars are critical for activity (13, 16, 24, 30). For example, the tomato steroidal glycoalkaloid α-tomatine has a tetrasaccharide chain attached to carbon 3 (Fig. 1A). A number of fungal pathogens of tomato produce enzymes that hydrolyze sugars from α-tomatine (collectively known as tomatinases) (reviewed in reference 30). Some of these remove just one sugar, while others hydrolyze all four sugars to give the aglycone tomatidine (Fig. 1A). The removal of sugars from saponins is traditionally associated with a reduction in antimi-

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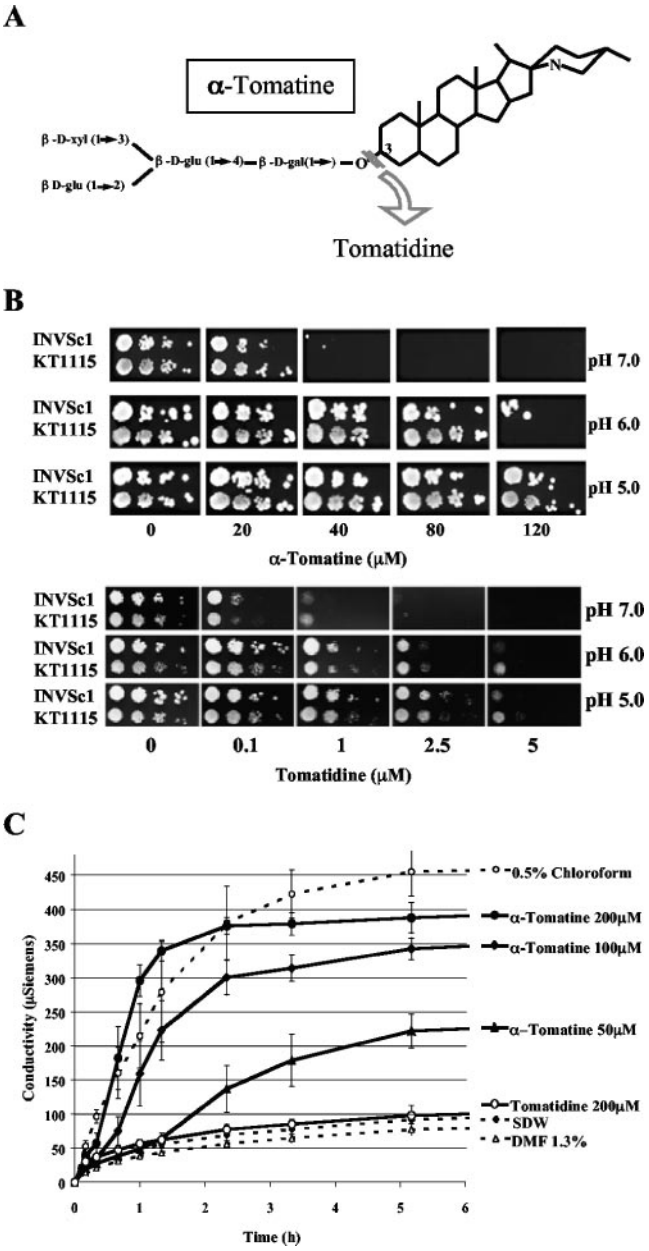


FIG. 1. Differential effects of α-tomatine and tomatidine on *Saccharomyces cerevisiae*. A. Structure of the tomato leaf saponin α-tomatine. The site of cleavage by fungal extracellular enzymes to yield the aglycone tomatidine is indicated. B. The sensitivities of wild-type *S. cerevisiae* strains INVSc1 and KT1115 to α-tomatine and tomatidine were measured in agar plate assays. The strains were pregrown in YEPD and the cell densities adjusted to 2×10^7 cells/ml. This cell suspension and 1:10, 1:100, and 1:1,000 dilutions were replica plated onto YEPD containing different concentrations of α-tomatine or tomatidine. Growth tests were carried out at a range of pH values, since the antifungal activity of α-tomatine is pH dependent. C. Electrolyte leakage measurements. Cells of *S. cerevisiae* strain INVSc1 were suspended in distilled water (5×10^8 cells/ml) and treated with α-tomatine, tomatidine, a solvent control (DMF), or a lysis control (chloroform). Conductivity, which is a measure of leakage of electrolytes from the cell (cell lysis), was measured over time. Mean values for three independent experiments are presented, with bars indicating standard error values.

TABLE 1. *Saccharomyces cerevisiae* strains used in these experiments

Strain	Genotype	Reference/ source
INVSc1	*MAT*α *his3*Δ*1 leu2 trp1-289 ura3-52*	Invitrogen
KT1357	*MAT***a** *leu2 ura3-52 his3 trp1-1*	15
KT1358	*MAT*α *leu2 ura3-52 his3 trp1-1*	6
KT1115	*MAT*α *leu2-3 leu2-112 ura3-52*	10
LPY27	*MAT***a** *erg2-4*::*LEU2 leu2 his3 ura3-52*	37
LPY25	*MAT***a** *erg3*::*LEU2 leu2 his3 ura3-52*	37
LPY11	*MAT***a** *erg6*::*LEU2 leu2 his3 ura3-52*	37
S288C	*MAT***a** *gal2*	31
X2180-1A	*MAT***a** *SUC2 mal0 gal2 CUP1*	28
UPC20	*MAT***a** *SUC2 mal0 gal2 CUP1 upc2-1*	28

crobial activity (30). However, α-tomatine hydrolysis products are able to suppress induced plant defense responses, indicating that they have other as yet uncharacterized effects on plant cells (7, 25). α-Tomatine and its hydrolysis products have also been associated with a variety of effects on human health, including toxicity, cholesterol lowering, enhanced immune responses as cancer chemotherapy agents, and protection against pathogenic fungi and other microorganisms (16). The biological mechanisms underpinning the varied and diverse effects of these compounds on microbes, plants, and animals are not yet understood.

Budding yeast (*Saccharomyces cerevisiae*) is an established model organism for investigation of the modes of action of antifungal and therapeutic compounds (1, 4, 18, 29, 42). It has also been used to study the biological properties of defensins, another class of molecule that play a role in plant defense against fungal attack (43, 44). Here we have exploited *S. cerevisiae* to investigate the effects of α-tomatine and tomatidine on eukaryotic cells.

MATERIALS AND METHODS

Yeast strains and media. The *S. cerevisiae* strains used are listed in Table 1.

Reagents. Stock solutions were made in dimethylformamide (DMF) or dimethyl sulfoxide (DMSO) as indicated. α-Tomatine, tomatidine, and nystatin were purchased from Sigma (Gillingham, Dorset, United Kingdom) and flutriafol and fenpropimorph from Riedel-de Haën (Seelzen, Germany).

Assays of antifungal activity. Yeast strains were tested for sensitivity to antifungal agents in agar plate growth tests on yeast extract-peptone-dextrose (YEPD) agar. Where necessary, the pH of the agar was adjusted with either HCl or NaOH prior to autoclaving. Serial dilutions from overnight cultures were plated onto YEPD agar supplemented with antifungal agents or solvent alone using a steel-pronged replicator. The plates were incubated at 30°C for two days and growth was assessed.

Electrolyte leakage experiments. The yeast strain INVSc1 was grown to mid log-phase in YEPD and the cells harvested by centrifugation at $200 \times g$ for 5 min, washed in sterile distilled water, and divided into aliquots of approximately 4×10^9 cells. After further centrifugation, the pellets were resuspended in sterile distilled water containing α-tomatine, tomatidine, or nystatin. The conductivity of the cell suspension was monitored with a Jenway 4010 conductivity meter (Jenway, London, United Kingdom) over a period of 6 h. Full lysis was monitored by incubation with chloroform (0.5% [vol/vol]) or nystatin (100 μM). Control treatments consisted of solvent alone (0.7% DMSO).

Gene expression analysis. RNA was prepared from yeast cells that had been treated with α-tomatine, tomatidine, or fenpropimorph at concentrations that gave 10 to 20% growth inhibition or with DMF solvent alone. An overnight culture of strain S288C was diluted to an optical density at 600 nm (OD_{600}) of 0.1. After 1 h of growth at 30°C, the compounds (or solvent alone) were added and the cultures were incubated for an additional 5 h. Growth inhibition was monitored by measuring the OD_{600}. Treatments were carried out in triplicate.

Cells were pelleted and frozen in liquid nitrogen. Acid-washed glass beads (0.5-mm diameter; Sigma) were added and the cells disrupted using two 20-s cycles at speed setting 6 in the Savant Bio 101 Fast Prep FP120. Total RNA was isolated using the RNeasy kit (QIAGEN, Inc., Valencia, CA). Each of the 12 RNA samples was then hybridized to GeneChip yeast genome S98 arrays (Affymetrix Inc., Santa Clara, CA) following the manufacturer's instructions. Data were analyzed using the RMA algorithm (GeneData Inc., Switzerland) and genes annotated according to the *Saccharomyces* Genome Database. An analysis of variance of the RMA expression values (log scale) was conducted for each gene, and gene expression for each of the three compounds was compared with that of the untreated control. A Bonferroni multiple-testing correction was applied to the contrast *P* value for each gene in order to minimize false positives.

Northern blot analysis was carried out by following standard procedures. PCR products for use as probes were obtained with gene-specific primers based on the coding regions of the respective genes, purified using a QIAquick nucleotide removal kit (QIAGEN, Crawley, United Kingdom) and radiolabeled with [α-^{32}P]dCTP by using a random prime-labeling system (Rediprime II; Amersham). Hybridization of blots was carried out at 65°C, and filters were washed at 65°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 0.1% sodium dodecyl sulfate.

Sterol analysis. Yeast strain S288C was grown in YEPD medium to saturation. The culture was diluted 15-fold and duplicate aliquots were treated with α-tomatine, tomatidine, or solvent alone. Cells were harvested when the control culture reached an OD_{600} of 1.2. The concentrations of inhibitory compounds used caused 20 to 30% growth inhibition. Freeze-dried cells were lysed at room temperature by overnight incubation with 80% ethyl alcohol–6% KOH (wt/vol). Sterols were extracted into chloroform and analyzed by gas chromatography/mass spectrometry (GC/MS) as previously described (27).

Microarray data accession number. Transcriptome data have been lodged with NCBI GEO under data deposit number GSE4669.

RESULTS

Inhibition of yeast growth by α-tomatine and its aglycone tomatidine is via distinct modes of action. Removal of sugars from saponins is traditionally associated with a loss of biological activity (16, 24, 30). We tested the inhibitory activity of α-tomatine (Fig. 1A) and its deglycosylated forms and found that intermediates lacking either of the terminal sugars (xylose or glucose) have little or no detectable antifungal activity, as expected (data not shown). Surprisingly, however, we found that the aglycone tomatidine is a far more potent antifungal agent than α-tomatine (Fig. 1B). Previous studies with filamentous fungi indicate that α-tomatine is more toxic at higher pHs when it is in its unprotonated form (16, 30, 41). Our experiments confirm this and in addition show that the inhibitory activity of tomatidine is also pH dependent (Fig. 1B). Yeast growth was completely inhibited by 0.1 to 1.0 μM tomatidine at pH 7, while the concentration of α-tomatine required for complete inhibition at this pH was substantially higher (20 to 40 μM) (Fig. 1B).

The inhibitory effects of saponins towards filamentous fungi are generally ascribed to the ability of these molecules to permeabilize membranes (24, 30). Our electrolyte leakage experiments show that α-tomatine causes dosage-dependent permeabilization of yeast cells (Fig. 1C). In contrast, tomatidine does not induce electrolyte leakage even at 200 μM, a concentration well in excess of that required for full inhibition of growth (Fig. 1B). These data indicate that α-tomatine and tomatidine have distinct modes of action.

Genome-wide expression profiling of the response of yeast to tomatidine treatment. Analysis of changes in gene expression in response to treatment with antimicrobial agents can yield critical information about likely modes of action and cellular targets (1, 4) and is emerging as a powerful tool in modern chemical genetics. We used genome-wide gene expression profiling to investigate the effects of tomatidine on yeast. Preliminary microarray experiments suggested that tomatidine might affect sterol biosynthesis (data not shown); we therefore included the sterol biosynthesis inhibitor fenpropimorph as a control in further experiments. In three independently replicated experiments, yeast cultures were treated with α-tomatine, tomatidine, or fenpropropimorph at concentrations selected to give 10 to 20% growth inhibition. RNA was recovered and transcriptional changes were assessed using Affymetrix whole genome microarrays. Genes with ≥1.5-fold mean changes in transcription and a Bonferroni-corrected *P* value of <0.05 were identified as having significant differences in expression. This analysis identified 271 genes showing differential expression between at least one of the inhibitor treatments and the control (see Table S1 in the supplemental material). Of these,

TABLE 2. Genes upregulated in response to tomatidine[a]

Functional class	Gene	Upc2p regulation[b]	Change in expression (*n*-fold)[c]	
			Tomatidine	Fenpropimorph
Cell wall mannoprotein	*DAN1*	+	33.87	75.99
	DAN4	+	10.50	32.95
	TIR3	+	4.10	6.92
	TIR4	+	4.86	20.66
	YMR317W	+	5.87	23.97
Sterol metabolism	*HES1*	+	9.04	34.72
	ATF2	+	3.75	3.40
	CYB5	+	2.94	3.11
	ERG3	+	2.22	2.44
	ERG28		2.32	3.51
	ERG5		1.82	2.58
	ERG25	+	1.67	2.19
	ERG4		1.68	1.52
Vesicular transport	*SRO77*	+	1.95	3.42
Amino acid metabolism	*CPA2*		3.10	1.13*
	MET17		2.14	3.39
	HOM3		1.65	1.07*
	ECM17		1.58	2.03
Phosphate metabolism	*PHO11*		3.39	1.72*
	PHO5		2.43	0.94*
	PHO8		1.77	1.44*
Cell cycle	*SCM4*		1.89	3.06
	PCL5		1.84	1.41*
Mitochondrial function	*DIC1*		2.17	1.92*
	YMC2		1.72	0.93*
Unknown function	*YPL272C*	+	5.96	29.14
	YGR131W	+	3.31	19.82
	YGL117W		2.95	1.19*
	YHR029C		2.02	1.76

[a] The 29 genes that were significantly upregulated in response to tomatidine (≥1.5-fold mean change in expression; Bonferroni multiple correction, $P < 0.05$) are listed. Values for fenpropimorph treatments are included for comparison. Genes that are known to be positively regulated by Upc2p are indicated (46).

[b] +, upregulation present.

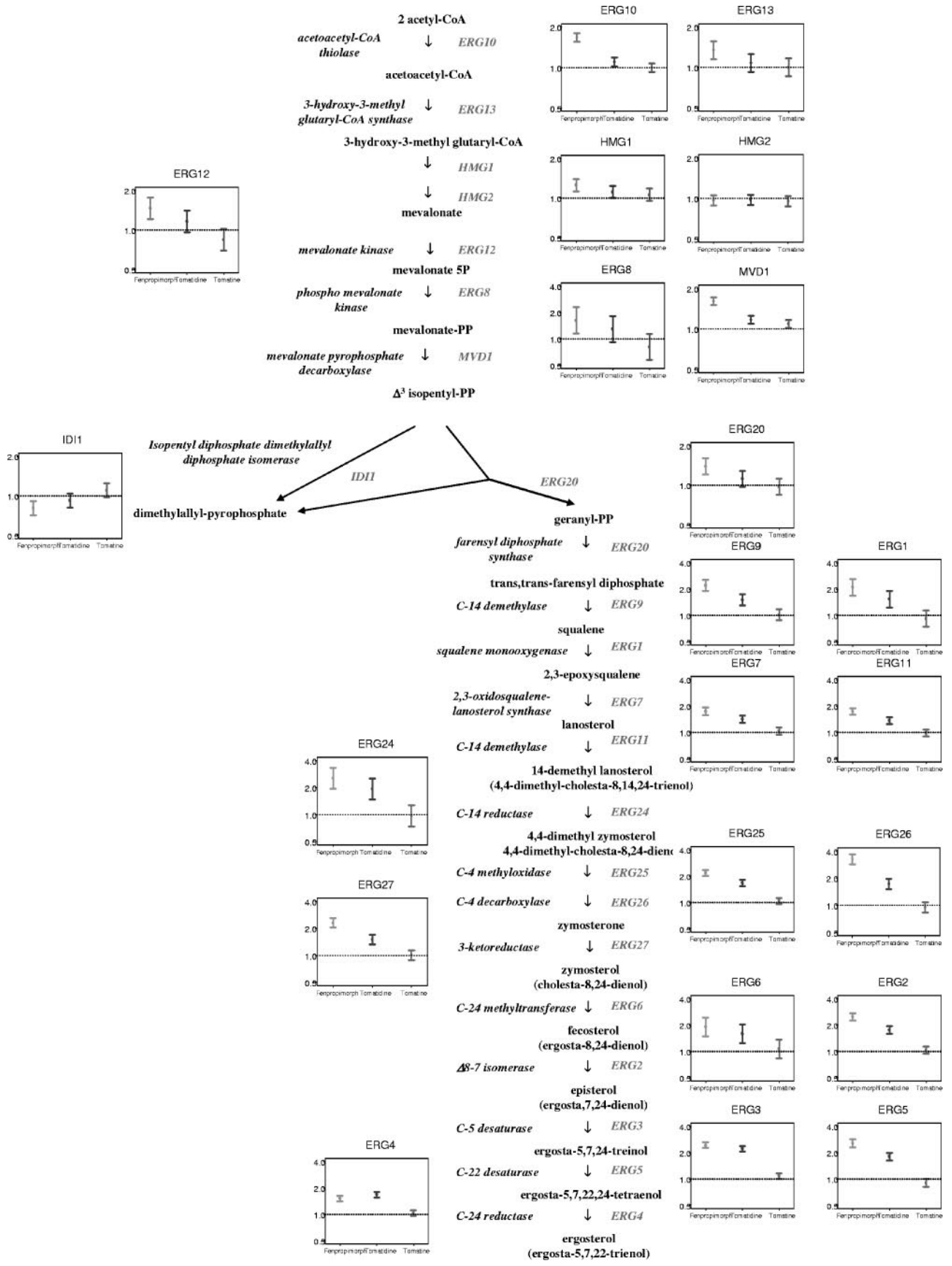
[c] *, not significantly different from the control. The full data set is presented in Table S1 in the supplemental material, and the complete transcriptome data set is lodged with NCBI GEO (data deposit number GSE4669).

TABLE 3. Response of Upc2p-regulated genes to fenpropimorph or tomatidine[a]

Gene	Response		Gene	Response	
	Fen	Tom		Fen	Tom
DAN/PAU/TIR family			*RIB5*	+	+
DAN1	+	+	*THI7*	+	+
DAN4	+	+	*ADE4*	+	−
TIR4	+	+			
DAN3	+	+	Carbohydrate metabolism		
TIR3	+	+	*AMS1*	+	+
YGL261C	+	+	*HXT3*	+	+
PAU7	+	−	*ARA1*	+	−
PAU2	+	+			
YGR294W	+	+	Vesicular/protein transport		
PAU1	+	+	*STS1*	−	−
YHL046C	+	+	*SAR1*	−	−
RNP1	−	−	*SRO77*	+	+
YDR542W	+	−			
PAU4	+	+	Protein modification		
PAU6	+	+	*KTR1*	+	+
YMR317W	+	+	*KTR6*	−	−
YIR041W	+	+	*SHG1*	−	−
YIL176C	+	−			
YAL068C	+	−	Energy generation		
PAU5	+	−	*MAM33*	+	+
YOL161C	+	−	*RSM26*	+	−
DAN2	+	+			
YLL025W	+	−	Protein synthesis		
YMR325W	+	−	*RPS10B*	−	−
TIR1	+	+	*RPS6A*	−	−
			YOL031C	−	−
Sterol metabolism			*YGR201C*	+	−
HES1	+	+	*GCD7*	+	−
ATF2	+	+			
NMD2	+	−	Other		
ERG3	+	+	*SCM4*	+	+
ERG24	+	+	*TSA2*	+	+
ERG26	+	+	*RBL2*	−	−
ERG25	+	+	*YPR204W*	−	−
ARE1	+	+	*PRM1*	−	−
CYB5	+	+	*PRM4*	+	+
NCP1	+	+	*PRY1*	−	−
			RIM21	+	−
Transport			*CDC42*	+	−
AUS1	+	+			
PDR11	+	+	Unknown		
FIT2	+	−	*YPL272C*	+	+
SPF1	−	−	*YGR131W*	+	+
CTR1	−	−	*YJL213W*	+	+
			YER188W	+	+
Transcription			*YLR297W*	+	+
HAP1	+	+	*YHL026C*	+	−
UPC2	+	+	*YAR069C*	−	−
			PHO36	+	−
Heme biosynthesis			*SRL3*	+	+
HEM13	+	+	*YLL058W*	+	+
HEM14	+	+	*YPR157W*	−	−
			YOL137W	−	−
Metabolism			*YBR090C*	−	−
SAH1	+	−	*RRP1*	+	+

[a] The 87 genes previously reported to be regulated by Upc2p (46) were assessed for changes in gene expression in response to treatment with fenpropimorph (Fen) or tomatidine (Tom). Genes that show an increase using a 95% confidence interval are indicated with a plus sign and all others with a minus sign. Genes are assigned to functional groups by using the annotation of the *Saccharomyces* Genome Database (http://www.yeastgenome.org/).

29 showed an increase in expression in response to tomatidine and none showed reduced expression (Table 2). A survey of these genes revealed clear links with effects on sterol-related responses. Thirteen of these genes had previously been shown to be positively regulated by Upc2p, a transcription factor involved in the regulation of sterol biosynthesis and uptake (46), while 8 were involved in sterol biosynthesis and 20 were also upregulated by fenpropimorph. Sixteen of the 20 genes that were upregulated by both tomatidine and fenpropimorph were either regulated by Upc2p and/or involved in sterol bio-


2 acetyl-CoA
acetoacetyl-CoA thiolase
ERG10
acetoacetyl-CoA
3-hydroxy-3-methyl glutaryl-CoA synthase
ERG13
3-hydroxy-3-methyl glutaryl-CoA
HMG1
HMG2
mevalonate
mevalonate kinase
ERG12
mevalonate 5P
phospho mevalonate kinase
ERG8
mevalonate-PP
mevalonate pyrophosphate decarboxylase
MVD1
Δ3 isopentyl-PP
Isopentyl diphosphate dimethylallyl diphosphate isomerase
IDI1
ERG20
dimethylallyl-pyrophosphate
geranyl-PP
farensyl diphosphate synthase
trans,trans-farensyl diphosphate
C-14 demethylase
ERG9
squalene
squalene monooxygenase
ERG1
2,3-epoxysqualene
2,3-oxidosqualene-lanosterol synthase
ERG7
lanosterol
C-14 demethylase
ERG11
14-demethyl lanosterol (4,4-dimethyl-cholesta-8,14,24-trienol)
C-14 reductase
ERG24
4,4-dimethyl zymosterol
4,4-dimethyl-cholesta-8,24-dienc
C-4 methyloxidase
ERG25
C-4 decarboxylase
ERG26
zymosterone
3-ketoreductase
ERG27
zymosterol (cholesta-8,24-dienol)
C-24 methyltransferase
ERG6
fecosterol (ergosta-8,24-dienol)
Δ8-7 isomerase
ERG2
episterol (ergosta,7,24-dienol)
C-5 desaturase
ERG3
ergosta-5,7,24-treinol
C-22 desaturase
ERG5
ergosta-5,7,22,24-tetraenol
C-24 reductase
ERG4
ergosterol (ergosta-5,7,22-trienol)

synthesis. Similar trends in the magnitude of the changes were evident for both tomatidine and fenpropimorph treatment; for example, *DAN1*, *DAN4*, *HES1*, and *YPL272C* showed the largest changes in both treatments (Table 2).

The high frequency of Upc2p-regulated genes responding to tomatidine and fenpropimorph led us to specifically compare the data for the full set of 87 genes that had been identified by others as being Upc2p regulated (46). Using a 95% confidence interval as before but lowering the threshold by omitting the high-stringency multiple-sampling correction step, we found that expression of 79% and 56% of this subset of genes were increased in response to fenpropimorph and tomatidine treatments, respectively (Table 3). Interestingly, *UPC2* showed an increase in expression of 4.6-fold in fenpropimorph-treated cells and 1.73-fold in tomatidine-treated cells. A similar analysis was carried out for 22 sterol biosynthetic genes. In this case, 21 genes and 16 genes showed changes in expression in response to fenpropimorph and tomatidine, respectively (Fig. 2). Taken together, these data provide strong evidence that tomatidine inhibits sterol biosynthesis, so causing increased expression of *UPC2* and Upc2p-regulated genes. Upregulation of expression of the related sterol-responsive transcription factor Ecm22p (45) was not observed.

Northern blot analysis confirmed that *UPC2*, the sterol biosynthesis genes *ERG3* and *ERG26*, and the *DAN*/*PAU*/*TIR* genes *DAN1*, *PAU1*, and *TIR3* are all upregulated in response to tomatidine treatment (Fig. 3). In contrast, there was little difference between the α-tomatine treatments and the DMSO-treated control, although α-tomatine may cause modest increases in expression of *ERG3* and *ERG26*. The Northern blot experiments also confirmed previous studies showing that interference with ergosterol biosynthesis in yeast (by treatment with sterol biosynthesis inhibitors or by mutation of sterol biosynthesis genes) results in coordinate upregulation of genes in the sterol biosynthetic pathway (1, 4, 46). Treatment with ergosterol biosynthesis inhibitors that block different steps in ergosterol biosynthesis (the azole flutriafol and the morpholine fenpropimorph) resulted in upregulation of all genes tested (Fig. 3). A similar pattern was seen with the *upc2-1* mutant of yeast, which has a gain-of-function mutation (28). Five of the six genes tested were also upregulated in the sterol biosynthesis *erg6*, *erg3*, and *erg2* mutants (*PAU1* is not responsive in these mutants). These experiments confirm that the effects of tomatidine on gene expression in yeast closely resemble those associated with sterol biosynthesis inhibitor treatment and with the *upc2-1* mutation.

Effects of tomatidine treatment on sterol content. The data presented above are consistent with the hypothesis that tomatidine inhibits ergosterol biosynthesis. Ergosterol biosynthesis in yeast proceeds through a pathway from lanosterol to zymosterol and then to ergosterol via a number of well-defined enzymatic steps (Fig. 2). Deletion of genes encoding enzymes in

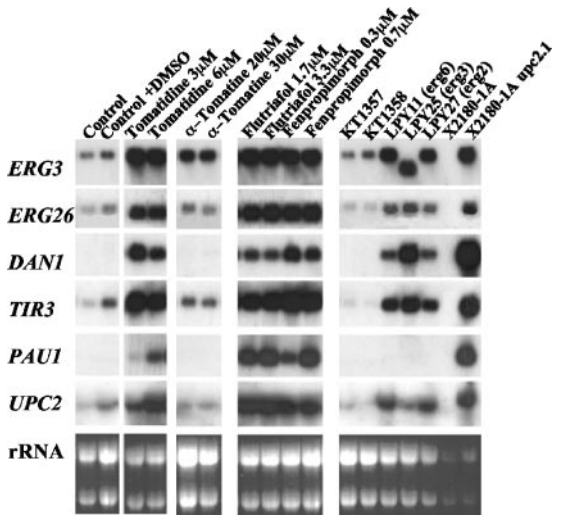


FIG. 3. Northern blot analysis of effects of chemical treatments and genetic mutations on gene expression. *S. cerevisiae* strain S288C was grown in the presence of α-tomatine, tomatidine, or the fungicides flutriafol and fenpropimorph. Controls included untreated yeast cells and cells grown in the presence of the solvent used to solubilize the antifungal compounds (1% DMSO). The *erg* mutants LPY11 (*erg6*), LPY25 (*erg3*), LPY27 (*erg2*) and the parent strains KT1357 and KT1358 were grown without drug treatment. The X2180-1A *upc2-1* mutant (UPC20) and corresponding wild-type strain X2180-1A were also included in these experiments. Strains were grown aerobically to mid-log phase in YEPD medium. Total RNA was extracted and analyzed by hybridization with probes specific for *ERG3*, *ERG26*, *DAN1*, *TIR3*, *PAU1*, and *UPC2*. The bottom panel indicates rRNA abundance as assessed by ethidium bromide staining.

the pathway between zymosterol and ergosterol does not block sterol synthesis but leads to an altered sterol profile in the mutant strain (2, 3, 17, 26). We used GC/MS analysis to investigate the effect of tomatidine treatment on sterol composition. The major sterol present in the control yeast cells was ergosterol, as expected (Fig. 4). The sterol profiles of α-tomatine-treated yeast cells were similar to that of the control. There was, however, a striking effect of tomatidine treatment on sterol composition. Tomatidine-treated yeast cells contained very little ergosterol and instead accumulated zymosterol (cholesta-8,24-dienol), providing strong confirmatory evidence that tomatidine blocks ergosterol biosynthesis. Accumulation of zymosterol is consistent with inhibition of the C_{24} methyltransferase encoded by *ERG6* (Fig. 2). The effects of tomatidine on yeast are likely to be more complicated than this, however, since tomatidine completely inhibits the growth of yeast, whereas deletion of *ERG6* is not lethal (17).

Sensitivity of sterol biosynthetic mutants. From the data presented above, we may expect yeast ergosterol biosynthesis mutants to show altered sensitivity to tomatidine. To test this,

FIG. 2. Effects of fenpropimorph, tomatidine, and α-tomatine on expression of ergosterol biosynthetic genes. The *S. cerevisiae* ergosterol biosynthetic pathway is shown along with the genes catalyzing each step. The expression levels of each gene in response to treatment with fenpropimorph, tomatidine, or α-tomatine were measured and the change (*n*-fold) relative to control treatment determined. Each graph shows this change with the bars indicating 95% confidence intervals. Any line that does not intersect unity (1.0) represents a statistically significant change in expression. Acetyl-CoA, acetyl coenzyme A.

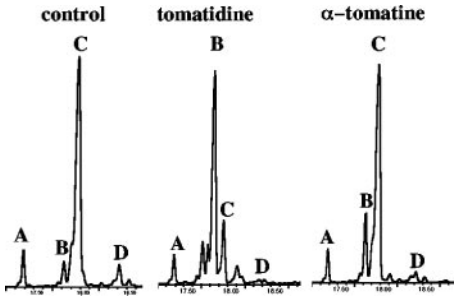


FIG. 4. Tomatidine inhibits sterol biosynthesis. Analysis of sterol content following treatment of yeast strain S288C with α-tomatine or tomatidine. Peaks: A, cholesterol (added as an internal standard); B, zymosterol; C, ergosterol; D, ergosta-5,7-dienol.

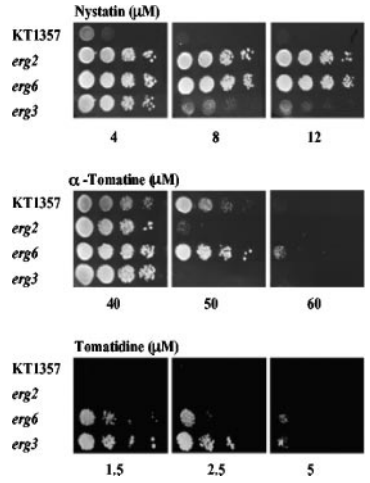


FIG. 5. Sensitivity of yeast sterol mutants to α-tomatine and tomatidine. The *S. cerevisiae* ergosterol biosynthetic mutants *erg2*, *erg6*, and *erg3*, as well as parent strain KT1357, were grown on agar plates (YEPD, pH 6.5) containing the membrane pemeabilizing agent nystatin, α-tomatine, or tomatidine as previously described for Fig. 1. All three mutants showed enhanced resistance to nystatin whereas differential effects were observed for tomatidine and α-tomatine.

erg2, *erg3*, and *erg6* mutants (37) were assessed for sensitivity to α-tomatine and tomatidine (Fig. 5). Clear differences were observed. The *erg3* and *erg6* mutants had enhanced resistance to tomatidine, while the *erg2* mutant showed wild-type sensitivity. All three mutants were resistance to nystatin as shown previously (37) but differed in sensitivity/resistance to α-tomatine, depending on the particular genetic lesion. Similar results were obtained with a second set of *erg* mutants derived from a different parent strain (23, 32), indicating that these effects are not specific to the particular genetic background of the parental strain (data not shown). These data suggest a complex interaction between the effects of *erg* mutations and tomatidine resistance/sensitivity that may be attributable in part to interactions of these compounds with different membrane sterols.

DISCUSSION

Biological activity of saponins is normally defined in terms of ability to complex with sterols, permeabilize membranes, or inhibit the growth of fungi (24). Removal of one or more sugars from the C_3 sugar chain is generally associated with loss of biological activity and/or detoxification (30). However, tomatidine and other α-tomatine hydrolysis products can interfere with induced defense responses in plants, indicating that these compounds also have biological activity of some kind (7, 25). Here we have shown that tomatidine (the aglycone of α-tomatine) has potent antifungal activity towards yeast and that this activity does not involve membrane permeabilization. Using a combination of gene expression analysis and GC/MS, we have demonstrated that tomatidine (but not α-tomatine) mimics the effects of sterol biosynthesis inhibitors. Tomatidine-treated cells accumulate zymosterol rather than ergosterol, which is consistent with inhibition of the sterol C_{24} methyltransferase Erg6p (5, 17, 32). There may be additional targets, however, since tomatidine completely inhibits the growth of yeast, whereas the deletion of *ERG6* is not lethal, and tests of the sensitivity of *erg* mutants to tomatidine indicate that the differential effects of this compound on different *erg* mutants are complex. In summary, our experiments indicate that tomatidine has a distinct mode of action to that of α-tomatine and that it targets the sterol biosynthetic pathway. This property appears to be a feature of steroidal alkaloid aglycones since other steroidal alkaloid aglycones such as solanidine were also inhibitory while the triterpene aglycones that we tested (oleanic acid, β-amyrin, and hederagenin) were not (data not shown). Future experiments to determination the precise mode of action of tomatidine will include isolation of mutants with altered tomatidine sensitivity.

The ergosterol biosynthetic pathway is a key target for chemical control of fungal pathogens of plants and animals, and there is constant pressure to identify new compounds with novel modes of action to combat the development of resistance to existing fungicides (14, 34, 48). The major fungicides that target sterol biosynthesis are azoles (Erg11p), morpholines (Erg24p, Erg2p, and Erg5p), and allylamines (Erg1p). Fungicides that target Erg6p (sterol C_{24} methyltransferase) are not in agricultural or clinical use although this enzyme can be inhibited by azasterols and other substrate analogues (33, 35, 39, 40). Interestingly, the deletion of *ERG6* increases the rate of passive drug diffusion in yeast, making the cells more susceptible to a broad range of chemicals (12). Thus, compounds that target Erg6p are likely to have a dual effect on fungi by inhibiting sterol biosynthesis and by facilitating uptake of other antimicrobial compounds. This has clear relevance for natural situations, since synergism between antifungal plant compounds is a well-known phenomenon. It also has direct relevance for strategies for control of pathogens in agriculture and in the clinic that involve use of combinations of chemicals. *erg6* mutants are hypersensitive to the triterpene saponin avenacin A-1, which is consistent with this prediction (our unpublished data). However, they have enhanced resistance to tomatidine and α-tomatine (Fig. 5).

Steroidal alkaloids are found in a variety of Solanaceous plants, both as precursors in the synthesis of steroidal glycoalkaloid saponins and as secondary metabolites per se. They can also be generated as a consequence of hydrolysis of steroidal glycoalkaloid saponins by fungal pathogens, and this may result in interference with induced defense mechanisms (7). From this study, the strategies employed by certain fungal pathogens

of tomato, such as *Fusarium oxysporum* f. sp. *lycopersici* of "detoxifying" α-tomatine to tomatidine (30), would seem to be counterintuitive, since tomatidine is a highly toxic metabolite. However, a previous study involving assessment of the relative toxicity of α-tomatine, β_2-tomatine (α-tomatine lacking the terminal glucose), and tomatidine to a range of pathogenic and nonpathogenic fungi has shown that fungal pathogens of Solanaceous plants are in general more resistant to tomatidine than nonpathogens (41). The mechanism of this resistance is not known. Nevertheless, the ability of *F. oxysporum* f. sp. *lycopersici* to withstand the toxic effects of tomatidine may confer a competitive advantage over other fungi that attempt to inhabit the same Solanaceous host plant, particularly since tomatidine may predispose sensitive microbes to the antimicrobial effects of other low-molecular-weight compounds. Importantly, tomatidine has recently been shown to suppress induced defense responses in suspension-cultured tomato cells (25). Future work that addresses the physiological effects of α-tomatine and its hydrolysis products on plant cells is expected to shed light on signaling processes associated with the establishment of plant-fungus interactions and on the relationship of these to sterol homeostasis.

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